

# Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/US05/009391

International filing date: 22 March 2005 (22.03.2005)

Document type: Certified copy of priority document

Document details: Country/Office: US  
Number: 60/555,167  
Filing date: 22 March 2004 (22.03.2004)

Date of receipt at the International Bureau: 30 May 2005 (30.05.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)



World Intellectual Property Organization (WIPO) - Geneva, Switzerland  
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**APPLICATION NUMBER: 60/555,167**

**FILING DATE: *March 22, 2004***

**RELATED PCT APPLICATION NUMBER: *PCT/US05/09391***



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EV362100395US

INVENTOR(S)						
Given Name (first and middle [if any])		Family Name or Surname		Residence (City and either State or Foreign Country)		
James Chanivan		Eshleman Shi		Lutherville, MD Baltimore, MD		
Additional inventors are being named on the _____ separately numbered sheets attached hereto						
TITLE OF THE INVENTION (500 characters max)						
A Method to Convert Single Base Changes to Large DNA Differences for Accurate and Sensitive Detection						
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<input checked="" type="checkbox"/> Specification Number of Pages		60		<input type="checkbox"/> CD(s), Number _____		
<input type="checkbox"/> Drawing(s) Number of Sheets		_____		<input type="checkbox"/> Other (specify) _____		
<input type="checkbox"/> Application Data Sheet. See 37 CFR 1.76						
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Respectfully submitted,

[Page 1 of 2]

SIGNATURE

TYPED or PRINTED NAME Heather Bakalyar, Ph.D.

TELEPHONE 410-516-8300

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**U.S. Provisional Patent Application**

**JHU Ref. No. DM-4354**

**A Method to Convert Single Base Changes to Large DNA  
Differences for Accurate and Sensitive Detection**

**Inventors:  
James Eshleman  
Chanjuan Shi**

All publications, patents and patent applications disclosed herein are incorporated into this application by reference in their entirety.

For example: "Sambrook et al, Molecular Cloning, A Laboratory Manual (volumes I-III) 1989, Cold Spring Harbor Laboratory Press, USA" and "Harlowe and Lane, Antibodies a Laboratory Manual 1988 and 1998, Cold Spring Harbor Laboratory Press, USA" provide sections describing methodology for antibody generation and purification, diagnostic platforms, cloning procedures, etc. that may be used in the practice of the instant invention.

The following claim(s) of this provisional application are not to be construed as limiting the disclosed invention(s). The claim(s) are included for compliance with patent application structural regulations that may be imposed by international patent offices.

We claim:

1. A method of detecting mutations in DNA comprising converting four or fewer base changes to large DNA differences.

## INVENTION DESCRIPTION

Describe the invention completely, using the outline given below. Please provide an **Electronic Copy** of the invention disclosure document, references, and abstracts, in Windows format, on CD-Rom or Floppy Disk.

**1. Abstract of the Invention** [In order to assist Licensing and Technology Development with the assessment of this technology, please provide a summary of the invention that should be written to be understood by a wide audience including non-technical individuals]

The method permits the sensitive and quantitative detection of point mutation or single nucleotide polymorphism (SNP) containing DNA. This is accomplished by converting the DNA containing a single base change into one that contains a completely unique target. This target is then detected in a highly quantitative and sensitive manner.

**2. Problem Solved** [Describe the problem solved by this invention]

A major problem in molecular analysis without current adequate solution is the accurate and sensitive detection of single base change containing DNA (base substitution mutations, SNPs, etc). Other methods to detect minor components of a DNA mixture (e.g. oligonucleotide ligation assay (OLA), allele-specific PCR (AS-PCR), amplification refractory mutation system (ARMS), standard realtime PCR (Q-PCR or RQ-PCR), among others), however the limit of detection for most of these methods is currently only between 1:10 and 1:100, which is insufficient for many applications.

This new invention permits relatively simple quantitative detection of small amounts of single base change containing molecules using a highly accurate method of detection and permitting a limit of detection of approximately 1:10,000 to 1:100,000, and possibly greater with additional modification.

**3. Novelty** [Identify those elements of the invention that are new when compared to the current state of the art]

This method uses a modification of Oligonucleotide Ligation Assay (OLA) to convert DNA molecules with only single base changes to those which contain a completely unique stretch of DNA (about 20 bases for use as probe detection). In the second part of the assay, realtime quantitative PCR (Q-PCR) is performed using universal primers to sensitively and quantitatively detect the probe containing DNA (and therefore the single base change). Because the strategy involves a ligation step, followed by a Q-PCR amplification step, we have designated the method as "LigAmp".

This method can be used for early detection of cancer, cancer minimal residual disease testing, HIV drug-resistant minority variant detection, identification of samples containing rare alleles and bone marrow transplantation engraftment monitoring.

#### 4. Detailed Description of the invention:

On a separate page(s), attach a detailed description of how to make and use the invention. The description must contain sufficient detail so that one skilled in the same discipline could reproduce the invention. Include the following as necessary:

- 1- data pertaining to the invention;
- 2- drawings or photographs illustrating the invention;
- 3- structural formulae if a chemical;
- 4- procedural steps if a process
- 5- a description of any prototype or working model;

In general, a manuscript that has been prepared for submission to a journal will satisfy this requirement.

**5. Workable Extent/Scope** [Describe the future course of related work, and possible variations of the present invention in terms of the broadest scope expected to be operable; if a **compound**, describe substitutions, breadth of substituents, derivatives, salts etc., if **DNA or other biological material**, describe modifications that are expected to be operable, if a **machine or device**, describe operational parameters of the device or a component thereof, including alternative structures for performing the various functions of the machine or device]

This is a general method with a wide range of potential applications. Adapting the system to detect different targets is accomplished by simply designing new oligonucleotides for the first step. Future related work will center on establishing proof-of-principal in a range of applications, including but not limited to: early detection of cancer, SNP/rare allele detection in pooled DNA, HIV drug resistance by detecting minor variants, detection of known paternal point mutations in maternal peripheral blood, and bone marrow engraftment analysis.

The second step of the process is universal. M13 forward and reverse primers are used in addition to two probes with no cross-reactivity to each other. Therefore one can easily adapt the system to different DNA targets to determine their absolute or relative concentrations.

**6. Key Words** [Please list specific key words that accurately describe the present invention]

Allele conversion, point mutation, single nucleotide polymorphism, ligation, realtime PCR, LigAmp.

**7. References** [Please list the closest and most relevant journal citations, patents, general knowledge or other public information related to the invention]

(For a full comparison of techniques, see the commercialization section below.)

Oligonucleotide ligation assay: Khanna, M., et al. Multiplex PCR/LDR for detection of K-ras mutations in primary colon tumors. *Oncogene*, 18: 27-38, 1999.

Southern blot: Mitsudomi, T., et al. Mutations of ras genes distinguish a subset of non-small-cell lung cancer cell lines from small-cell lung cancer cell lines. *Oncogene*, 6: 1353-1362, 1991.

Realtime PCR: Oliver DH, et al. Use of single nucleotide polymorphisms (SNP) and real-time polymerase chain reaction for bone marrow engraftment analysis. *J Mol Diagn*. 2000 Nov;2(4):202-8.

Allele-specific PCR/ARMS: Clayton, S. J., et al. K-ras point mutation detection in lung cancer: comparison of two approaches to somatic mutation detection using ARMS allele-specific amplification. *Clin Chem*, 46: 1929-1938, 2000. Germer, S., H, et al. High-throughput SNP allele-frequency determination in pooled DNA samples by kinetic PCR. *Genome Res*, 10: 258-266, 2000.



## SOFTWARE IMPLEMENTATION OF THE INVENTION

Indicate if this disclosure of invention is software or if software is implemented in the invention.

☐ Yes ☒ No

If Yes, describe the implementation of the software completely, using the outline given below.

**1. Scope of Work** [Is the work original? Is it created within the scope of your employment at JHU? Please explain the circumstances of program's development]

None ☒

**2. Software Developers** [Please list any developers of the software if different from invention]

None ☒

**3. Software Derivation** [If software is a derivative of an existing work, please explain the original work's source and the modification]

None ☒

**4. Third Party Content** [Identify any third party content or other elements and their source included in the software]

None ☒

**5. Brief Software Description** [Please characterize how robust and user friendly the work is.]

None ☒

## RESEARCH SUPPORT INFORMATION

Indicate **ALL** contributions to the development of the invention in terms of personnel, money; materials and facilities etc.

Check each funding source that applies to this invention:

☐ None    ☒ Federal Sponsor(s)    ☐ University Funding    ☐ Commercial Funding    ☐ Other

For each funding source, provide the below information. Additionally, if "Commercial" or "Other" Funding was used, please attach a copy of each such award notice.

<u>Granting/Funding Source</u>	<u>Award/Contract Number</u>	<u>Title of Grant</u>	<u>Copy Attached</u>
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## AGREEMENT SUPPORT INFORMATION

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If yes, please provide the following information for each item and attach a copy of the Agreement.

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## LigAmp: Sensitive Detection of Single Nucleotide Differences

Chanjuan Shi<sup>1</sup>, Li Hua<sup>1</sup>, Dana Jones<sup>1</sup>, Antony R. Parker<sup>1</sup>, Susan H. Eshleman<sup>1</sup> and James R. Eshleman<sup>1,2,3</sup>

Departments of <sup>1</sup>Pathology and <sup>2</sup>Oncology  
Johns Hopkins University School of Medicine

**Keywords:** allele conversion, oligonucleotide ligation assay, Q-PCR, Cepheid SmartCycler, real-time PCR, mutation, HIV-1, K-ras, minority variant

**Abbreviations:** Q-PCR, real-time quantitative PCR; PAGE, polyacrylamide gel electrophoresis, Ct, cycle threshold.

**Acknowledgements:** We acknowledge many helpful discussions with Brian Wendelburg (Cepheid) and Marsha Slater (Applied Biosystems). This work was supported by an R01 CA81439 grant from the National Cancer Institute (to J.R.E.), the HIV Prevention Trials Network (HPTN) sponsored by the NIAID, National Institutes of Child Health and Human Development (NICHD), National Institute on Drug Abuse, National Institute of Mental Health, and Office of AIDS Research, of the NIH, DHHS (U01-AI-46745 and U01-AI-48054), the Adult AIDS Clinical Trials Groups (NIH, Division of AIDS, NIAID), R01-HD042965-01 (To S.H.E.), and Canadian Institute of Health Research fellowship (to C.S.)

<sup>3</sup>. Correspondence:

James R. Eshleman, M.D., Ph.D.  
Associate Professor  
Department of Pathology  
Ross 632  
720 Rutland Ave.  
Baltimore, MD 21205  
[jeshlema@jhmi.edu](mailto:jeshlema@jhmi.edu)  
phone: 410-955-3511  
fax: 410-614-0671

**Abstract**

Sensitive detection and accurate quantification of small number of mutant viruses and cells is a challenge in many research and clinical applications. This has been difficult in part since the mutant DNA often differs from the wild-type DNA by only a single base. While real-time quantitative PCR (Q-PCR) can accurately detect DNA with an impressive linear range (7-10 orders of magnitude), it performs poorly for detection of single base changes because of probe cross-hybridization. We report a strategy that converts single base changes into larger DNA differences that can then be selectively detected in a sensitive and quantitative fashion. This strategy combines oligonucleotide ligation at a gene mutation site with subsequent Q-PCR amplification of the ligated products. The assay can reliably detect one mutant DNA molecule in the presence of a background of  $10^4$ - $10^5$  wild-type molecules. With this level of sensitivity and accurate quantification, this approach may find applications in cancer, infectious disease and genetic testing.

## Introduction

Single base mutations play a role in the pathogenicity of many human diseases, including cancer. Viral drug resistance is also commonly associated with single base mutations in viral genomes. In addition to their pathogenicity, point mutations can also be effective markers for DNA-based diagnostics. In many circumstances however, mutants co-exist with a large excess of wild-type cells or viruses. Therefore, development of sensitive and accurate strategies to detect single base differences is an important challenge.

Several strategies already exist to detect single base changes in genomic DNA, but their limit of detection is typically 0.1-1% (approximately 1 mutant cell among 100-1000 wild-type cells). Restriction length polymorphism (RFLP)/Southern blot assays typically have a limit of detection of approximately 0.5-5%<sup>1,2</sup>. The oligonucleotide ligation assay (OLA), even with sensitive capillary electrophoresis detection, can only detect minor species at about 0.1-1%<sup>3,4</sup>. With allele-specific PCR (AS-PCR) or the amplification refractory mutation system (ARMS), two separate PCR reactions are used, each with a forward PCR primer that is perfectly matched to either the wild-type or mutant allele at its 3' end<sup>5,6</sup>. However, even though Taq polymerase lacks proof-reading activity, the enzyme can still extend DNA from a mispaired primer, limiting the detection of AS-PCR and ARMS assays to a sensitivity range of about 1%<sup>7,8</sup>. We recently attempted to use Q-PCR and single nucleotide polymorphisms (SNPs) to monitor the level of bone marrow transplant engraftment in mixed chimera DNA samples, but were only able to attain a limit of detection of 5-10% due to cross-hybridization of the probes<sup>9</sup>. Other assays can detect single base mutations at lower levels<sup>10-13</sup>, however those methods

often rely on the presence of unique DNA sequences adjacent to the target mutations, and therefore cannot be generally applied.

The present study was designed to develop a universal strategy for accurate, sensitive detection and quantification of DNA containing point mutations.

### Materials and Methods

Oligonucleotides and probes: The K-ras and HIV-1 oligonucleotides (gel-purified), and M13 forward and reverse primers (Table 1) were purchased from Invitrogen, Corp. (Carlsbad, CA). The downstream common oligonucleotides were phosphorylated at the 5' end using T4 polynucleotide kinase (New England Biolabs, Beverly, MA). The *lacZ* and 16S rRNA Taqman probes containing different fluorophores and quenchers (Table 1) were purchased from Integrated DNA Technology (Coralville, IA).

Mutant K-ras oligonucleotide ligation: Wild-type (HeLa cells) and K-ras mutant (SW480 cells) genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Ten-fold serial dilutions of the mutant DNA (1.2 µg to 12 pg) were made in 1.2 µg wild-type DNA. For ligation, 1 pmol of the K-ras upstream mutant or wild-type oligonucleotide and 1 pmol of the K-ras common oligonucleotide were incubated with the DNA in ligase buffer containing 20 mM Tris-HCl (pH 7.5), 20 mM KCl, 10 mM MgCl<sub>2</sub>, 0.1% Igepal, 0.01 mM rATP, 1 mM DTT, and 4 U *pfu* DNA ligase (Stratagene, La Jolla, CA). The ligation conditions included denaturation at 95 °C for 3 min, followed by 90 cycles of 95 °C for 30 seconds and 65 °C for 4 min. Ligation reactions for wild-type and mutant alleles were performed in separate tubes, and used directly for Q-PCR without purification.

HIV-1 oligonucleotide ligation: Plasmids containing infectious molecular clones of HIV-1 DNA with and without the K103N drug resistance mutation were generously provided by Dr. John Mellors (Univ. of Pittsburgh). *Pol* region DNA was amplified from the

plasmids using the ViroSeq™ HIV-1 Genotyping System (ViroSeq, Celera Diagnostics, Alameda, CA) and was subcloned into the pCRII-TOPO® cloning vector using the TOPO TA Cloning® Kit, Version N (Invitrogen). The wild-type and mutant plasmids were isolated using a commercial kit (QIAwell Ultra Plasmid Kit (Qiagen) and sequenced using the ViroSeq system. Ten-fold serial dilutions of the mutant plasmid were prepared by mixing the mutant plasmid with the wild-type plasmid. Plasmid mixtures were prepared at a final concentration of 2 pg/μl. Ligation was performed using 10 pg of plasmid mix and the conditions listed above, except that 2 pmol upstream oligonucleotide and 8 units of the ligase were used.

Q-PCR: Q-PCR amplification of ligated products was performed using a SmartCycler (Cepheid, Sunnyvale, CA). Each reaction (25 μl) contained 5 pmol forward and 5 pmol reverse M13 primers, 6 μl of the unpurified ligation reaction, 12.5 μl platinum Quantitative PCR SuperMix-UDG (Invitrogen), and 2.5 pmol of either the *lacZ* or 16S rRNA probe. PCR reactions included pre-incubation at 50 °C for 2 min and 95 °C for 2 min, followed by 50 cycles of 95 °C for 10 seconds and 64 °C for 20 seconds. Following Q-PCR detection, PCR products were electrophoresed on a 4-12% acrylamide gel (Novex® TBE gel, Invitrogen) and stained with ethidium bromide.



## Results

Q-PCR is an exciting, relatively new method whereby PCR reactions are continuously monitored during amplification using a variety of different fluorescent probes. It has a remarkably wide dynamic linear range, but has been difficult to apply to point mutation detection because probes that differ by a single nucleotide often cross-hybridize to both the wild-type and mutant templates. We therefore sought a strategy that would convert these single base differences into more distinctive molecules. This strategy (Figure 1) employs two unique oligonucleotides. Each of these contains a region specific to the target gene and an M13 tail, which facilitates subsequent PCR amplification of the ligated product with universal M13 amplification primers. The 3' end of the upstream wild-type and mutant oligonucleotides perfectly match the corresponding sequences in the appropriate target molecule (if present). The upstream oligonucleotide also contains a region of unique foreign DNA that serves as the binding region for a universal probe in a subsequent detection reaction. The analysis can be performed as a single reaction to detect and directly quantify only the mutant DNA (Figure 1), or as a pair of reactions where both mutant and wild-type DNA are detected separately for relative quantification. In either case, the strategy relies on the differentiating power of a DNA ligase to ligate the oligonucleotides only when they are both fully hybridized to the template DNA (no mismatch is present at the adjacent terminal nucleotides). Q-PCR with universal M13 primers is used to amplify the ligated product. The resulting amplicons are then detected using a universal probe. Both the M13 forward primer and the *lacZ* probe have the same polarity as the upstream ligation oligonucleotide (Table 1). Therefore, the *lacZ* probe cannot bind to the ligation oligonucleotide. Binding of the

probe first requires successful ligation of the two oligonucleotides and polymerization of the complementary (bottom) strand of DNA by Taq polymerase. Subsequent extension of Taq polymerase from the M13 primer towards the bound *LacZ* probe allows the probe to be cleaved, and the fluorophore to be released and detected. Since this strategy involves a ligation step followed by an amplification/detection step, we have designated it, "LigAmp".

To demonstrate initial proof-of-principal, we used a colon cancer cell line, SW480, which contains a K-ras mutation (GGT to GTT, Gly12Val), and performed 10-fold serial dilutions of this mutant DNA into HeLa cell line DNA, which contains wild-type K-ras genes. After ligation of either the mutant or wild-type specific oligonucleotide to the common oligonucleotide, Q-PCR was performed using the universal M13 primers and *lacZ* probe. We then determined the cycle number at which threshold was achieved (Ct). Figure 2a shows that the mutant K-ras DNA can be easily detected as a signal distinct from pure wild-type DNA even when present at a level of only 1:10,000 (4-5 cycles difference in Ct). At a concentration of 1:100,000, the signal is still distinct from that of wild-type alone ( $p < 0.01$ , paired student *t*-test), though the difference was only 1-2 cycles. Detection of the K-ras mutant DNA was linear over the full range of dilutions (Figure 2b,  $r^2 = 0.99$ ). When the same amplified products shown in Figure 2a were separated by polyacrylamide gel electrophoresis (PAGE, Figure 2c), the intensity of the ethidium bromide-stained PCR product (106 bp) correlated well with the level of input mutant DNA, and correlated inversely with the Q-PCR cycle threshold, as expected.

As a second model system, we tested whether LigAmp could be used to detect minority variants within a population of HIV-1 viruses. We prepared mixtures of

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plasmids with wild-type (K=AAA) or mutant (N=AAC) codons at amino acid 103 in HIV-1 reverse transcriptase. The K103N mutation is associated with resistance to the non-nucleoside reverse transcriptase inhibitor (NNRTI) class of antiretroviral drugs used to treat HIV-1 infection. After preparing 10-fold serial dilutions of the mutant plasmid, we performed ligation with a mutant upstream and common downstream HIV-1 oligonucleotide (Table 1), and Q-PCR using the same M13 primers and *lacZ* probe (Figure 3). The Ct was determined as described above. Using this approach, we were able to detect the K103N mutation in the plasmid mixtures at a level of 1:10,000 (0.01%). The wild-type plasmid was not detected.

## Discussion

In this report we demonstrate that LigAmp, which converts nearly identical molecules into distinctive ones, is a highly sensitive and specific technique that can quantitatively detect low levels of DNA containing a single base mutation in the presence of a vast excess of wild-type DNA. A sensitivity of  $10^{-4}$  to  $10^{-5}$  was easily achieved and was reproducible. Based on previous estimates that each human cell contains approximately 6 pg genomic DNA<sup>11</sup>, the present assay can detect as few as 2-20 molecules of mutant K-ras in the presence of vast excess of normal genomic DNA. A low level non-specific signal was observed using wild-type DNA as template, but was clearly distinct from the  $10^{-4}$ - $10^{-5}$  dilution of the mutant DNA. This non-specific signal is generated during the ligation step, since this signal is not seen without the addition of ligated products. It presumably arises either because non-specific ligation still occurs across mispairs at a very low frequency, or because template-independent ligation occurs. The Q-PCR second step employs a probe that hybridizes to a foreign DNA sequence (e.g. *lacZ*) introduced into the ligated product. This minimizes the potential for detection of the wild-type sequence, making this method more specific than traditional Q-PCR based assays<sup>9</sup>.

Previously reported strategies that are highly sensitive for detection of point mutations (see Introduction) are generally more complex than LigAmp and less quantitative. Also, since no specific sequence context is required for the ligation step, and since the M13 primers and detection probes are universal in the Q-PCR step, this method should be easily adapted for any point mutation. In theory, one could perform the ligation

step as a multiplex reaction for simultaneous detection of several target genes, using multiple probes with unique fluorophores.

For many cancer diagnostic applications, one would like to detect specific mutations that are common to many cancer types (e.g. K-ras, p53, Braf mutations). Several exciting new initiatives with very large potential clinical impact in cancer research are underway. These include the creation of the Early Detection Research Network for early detection of common cancers both for families at increased risk and the general population<sup>14,15</sup>. Another exciting area of research is the use of molecular assays to detect cancer relapse, where a cancer patient in remission can be monitored for recurrence and re-treated when only a small number of cancer cells is detected using sensitive molecular strategies<sup>16</sup>. Finally, a sensitive point mutation detection strategy would permit the use of SNPs to monitor bone marrow transplant engraftment.

Technologies for ultra-sensitive detection of point mutations are potentially also important for management of patients with HIV-1 infection. HIV-1 infected patients typically harbor a swarm of genetically-related viral variants. Use of antiretroviral drugs to prevent and treat HIV-1 infection can select for drug-resistant variants that have point mutations in the HIV-1 protease and reverse transcriptase coding regions. Most HIV-1 genotyping assays used to identify drug resistance mutations are designed to detect only the major viral population, and have relatively low sensitivity for minority variants. A recent blinded, multi-center study compared the sensitivity of different methods to detect minority variants of HIV-1 with the K103N drug resistance mutation that were present at different levels in laboratory-prepared viral stocks<sup>17</sup>. Two allele-specific Q-PCR (AS-Q-PCR) assays and a yeast hybrid assay specific for HIV-1 reverse transcriptase could

detect the mutation at <1%. One AS-Q-PCR assay detected the mutation as low as 0.1%. Other assays that were less sensitive included an RT-PCR assay with allele-specific hybridization, two oligonucleotide ligation assays, single genome RT/PCR and sequencing, and RT/PCR with pyrosequencing, and RT/PCR with direct sequencing of the bulk population using two FDA-cleared HIV-1 genotyping assays. The interest in developing ultra-sensitive methods for detection of HIV-1 minority variants is growing, following the report of a study that suggests that the presence of such variants can influence treatment response<sup>18</sup>. Using plasmid mixtures, LigAmp was able to detect the K103N mutation in mixtures at a level of  $10^{-4}$  (0.01%). We are currently optimizing LigAmp for detection of minority drug resistance variants of HIV-1 directly in plasma samples.

LigAmp may also have applications in prenatal diagnosis. In that setting, it is often desirable to determine whether a known paternal point mutation has been transmitted to a fetus. Using LigAmp, it may be possible to detect such mutations in small numbers of fetal cells present in a wild-type mother's peripheral blood, thereby avoiding amniocentesis or chorionic villous sampling procedures that carry an approximate 0.5% risk of fetal loss<sup>19,20</sup>.

In summary, LigAmp is a universal point mutation detection strategy with many potential cancer, infectious disease and genetics applications.

Figure Legends

Figure 1: LigAmp Strategy. LigAmp involves two discrete steps. *a.* In the first step, a point mutation is converted by selective ligation into a molecule containing a unique DNA sequence (e.g. *lacZ*). Ligation oligonucleotides contain sequences homologous to the universal M13 Q-PCR amplification primers (blue), the universal *LacZ* Q-PCR probe (red), and the DNA template (green). If the terminal adjacent bases of the two ligation oligonucleotides hybridize fully to the template DNA (no mismatch), ligation occurs. *b.* In the second step, Q-PCR is performed using M13 forward and reverse primers combined with a probe that is unique to the ligation product. The M13 reverse primer first serves as a primer for polymerization of DNA complementary to the ligation product. This allows the M13 forward primer and *LacZ* probe to bind. Extension by Taq polymerase from the M13 forward primer leads to digestion of the probe with release and detection of the fluorophore. *c.* When wild-type DNA is used as a template, the mutant oligonucleotide should not ligate to the common downstream oligonucleotide due to nucleotide mis-pairing at the ligation site. *d.* Therefore, no subsequent amplification should occur and no signal should be detected in the Q-PCR step. One can use LigAmp for direct quantification of mutant DNA (as shown) or for relative quantification of mutant and wild-type (not shown) DNA.

Figure 2: Sensitive detection of DNA containing the K-ras mutation. *a.* Representative Q-PCR amplification curves (in duplicate) of K-ras mutant SW480 genomic DNA serially diluted into wild-type K-ras HeLa DNA. Note that 1:10,000 dilutions are clearly distinct from pure wild-type DNA. The 1:100,000 dilutions are also distinct ( $p < 0.01$ ). *b.* The

mean Ct values (four independent amplifications) were plotted against the relative concentration of DNA ( $r^2=0.995$ ). *c.* PAGE of the same DNA products produced in Q-PCR (Panel a). The "H<sub>2</sub>O + ligation" sample is a water control that was subjected to both ligation and PCR steps, while the "H<sub>2</sub>O" sample was only subjected to the PCR step. Data was analyzed using paired student t-test.

Figure 3: Detection of the K103N mutation in HIV-1 reverse transcriptase. *a.* Representative Q-PCR amplification curves (in duplicate) of K103N mutant plasmid serially diluted with the "wild-type" HIV-1 plasmid. Note that 1:10,000 dilutions of the mutant plasmid are clearly distinct from the pure wild-type plasmid, which doesn't achieve threshold. *b.* The mean Ct values (six independent amplifications) were plotted against the relative concentration of mutant plasmid ( $r^2=0.985$ ). *c.* PAGE of the DNA products produced in Q-PCR (Panel a).



Table 1. Oligonucleotides and probes

K-ras Ligation Oligos	Mutant K-ras upstream	5'- <u>ACTGTAAAACGACGGCCAGTGT</u> - <i>TCCCCTCAA</i> <u>ACTG</u> <i>GCAGATGCACG</i> -C-TTGTGGTAGTTGGAGCTGT*-3'
	WT K-ras upstream	5'-A- <u>CTGTAAAACGACGGCCAGTGT</u> - <i>CGTATTACCGCG</i> <i>GCTGCTGGCAC</i> -C-TTGTGGTAGTTGGAGCTGG*-3'
	K-ras common downstream	5'-PO <sup>4</sup> - <b>TGGCGTAGGCAAGAGTGCC</b> - <u>TGGTCATAGC</u> <u>TGTTTCCTGCA</u> -3'
HIV-1 Ligation Oligos	Mutant HIV-1 upstream	5'- <u>ACTGTAAAACGACGGCCAGTGT</u> - <i>TCCCCTCAA</i> <u>ACTG</u> <i>GCAGATGCACG</i> -CGCAGGGTTAAAAAG <u>GAC</u> *-3'
	HIV-1 common downstream	5'-PO <sup>4</sup> -AAATCAGTAACAGTACTGGATGTGGGTG- <u>TGGTCATAGCTGTTTC</u> CTGCA-3'
Amplification Primers	M13 forward	5'-CTGTAAAACGACGGCCAGTG-3'
	M13 reverse	5'-TGCAGGAAACAGCTATGACCA-3'
Probes	<i>lacZ</i> probe	FAM-5'-TCCCCTCAA <u>ACTGGCAGATGCACG</u> -3'-BHQ-1
	<i>16S rDNA</i>	ROX-5'-CGTATTACCGCGGCTGCTGGCAC-3'-BHQ-2

Underlined: M13 primer binding regions. Italics: probe binding regions. Bold: K-ras-specific or HIV-1-specific regions. Asterisk: terminal bases with perfect homology to either the wild-type or mutant K-ras or HIV-1 sequences. FAM: 6-carboxyfluorescein.

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ROX: 6-carboxy-X-rhodamine. BHQ: black hole quencher. Boxed base: An additional mis-pair in the upstream HIV-1 mutant oligonucleotide at the third base from the 3' end was introduced to improve the specificity of the assay (the usual mutant 3' terminal sequence is GAAC).

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Fig 1. Shi et al.

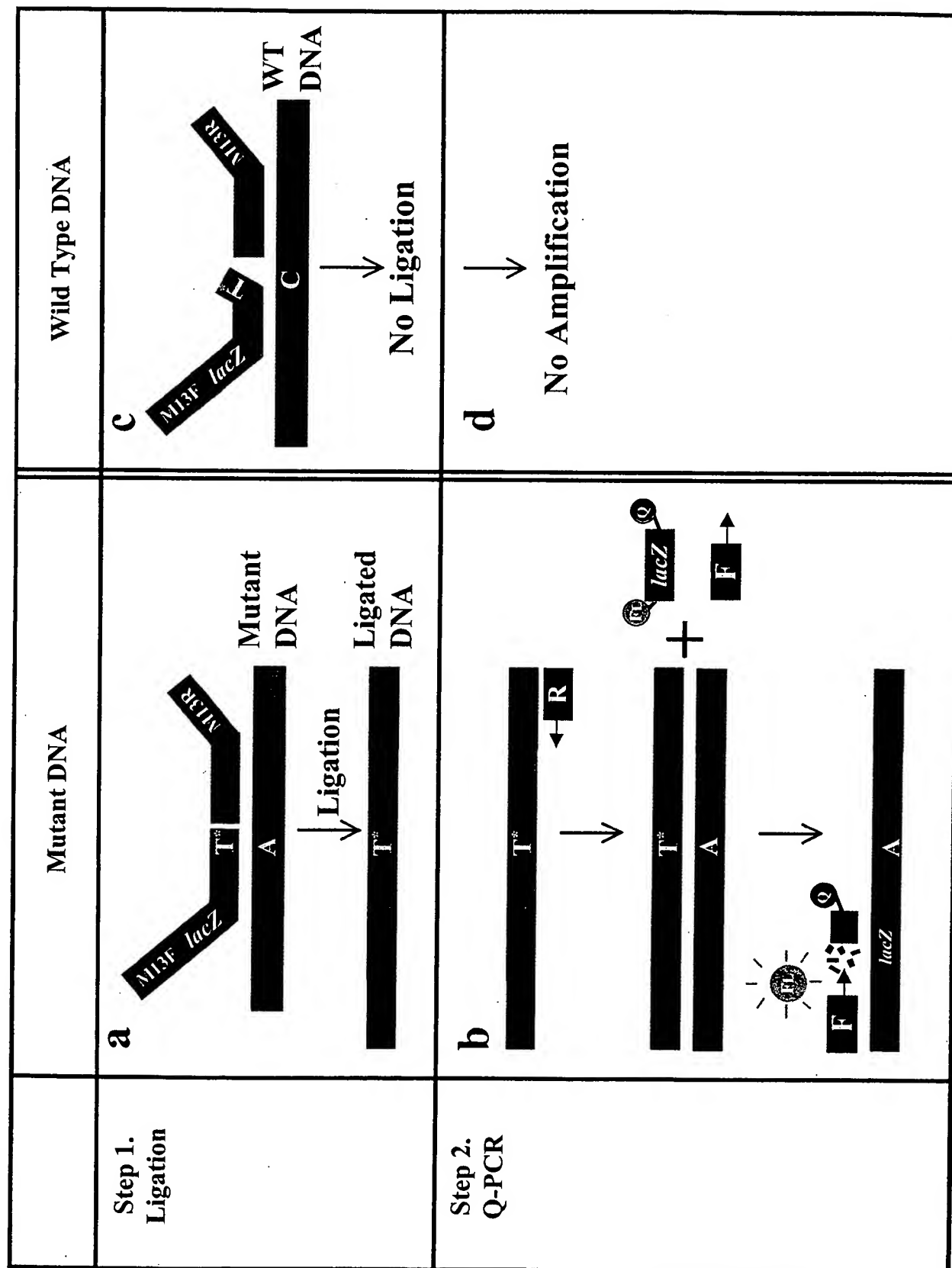


Fig 2. Shi et al.

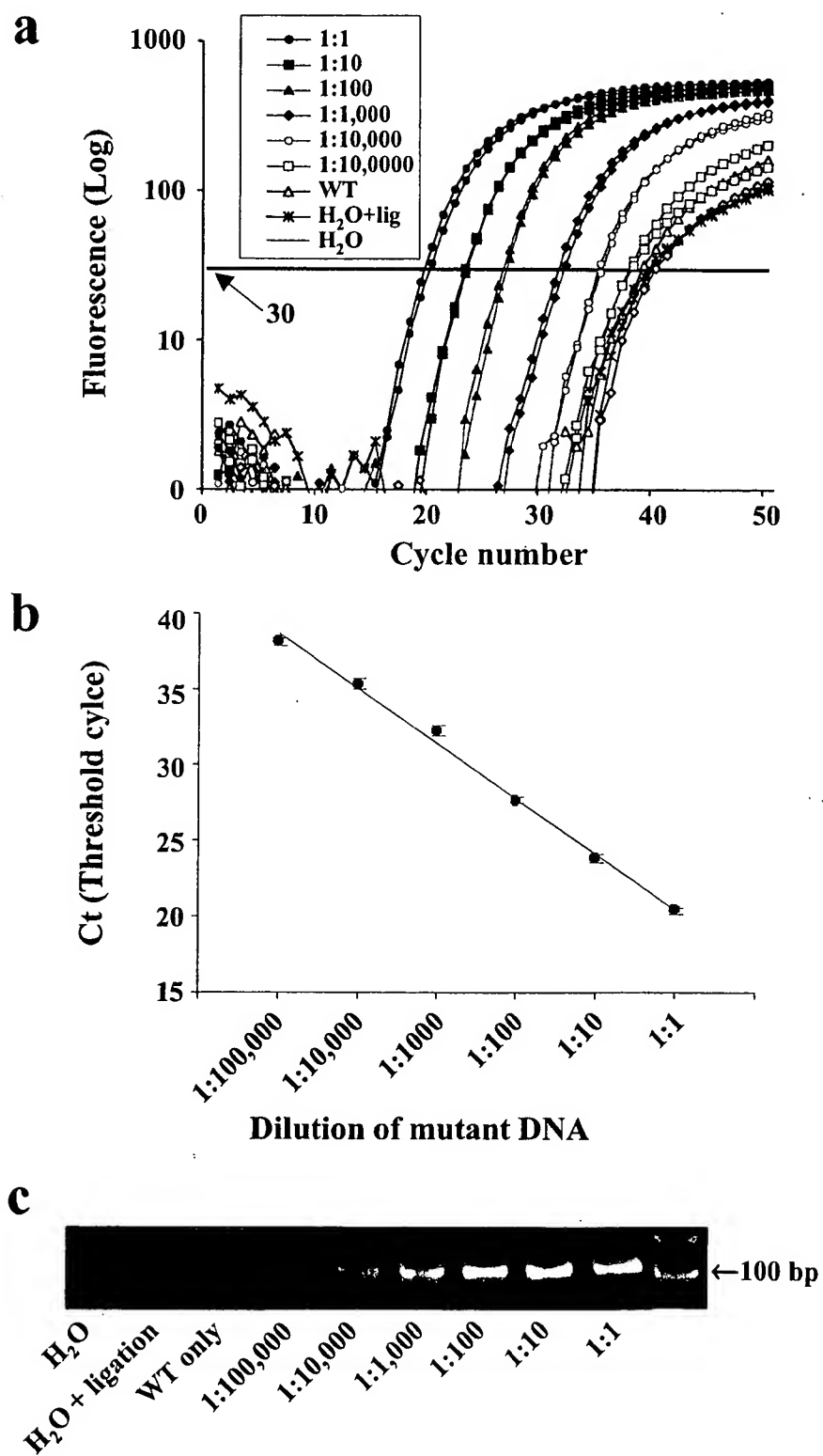
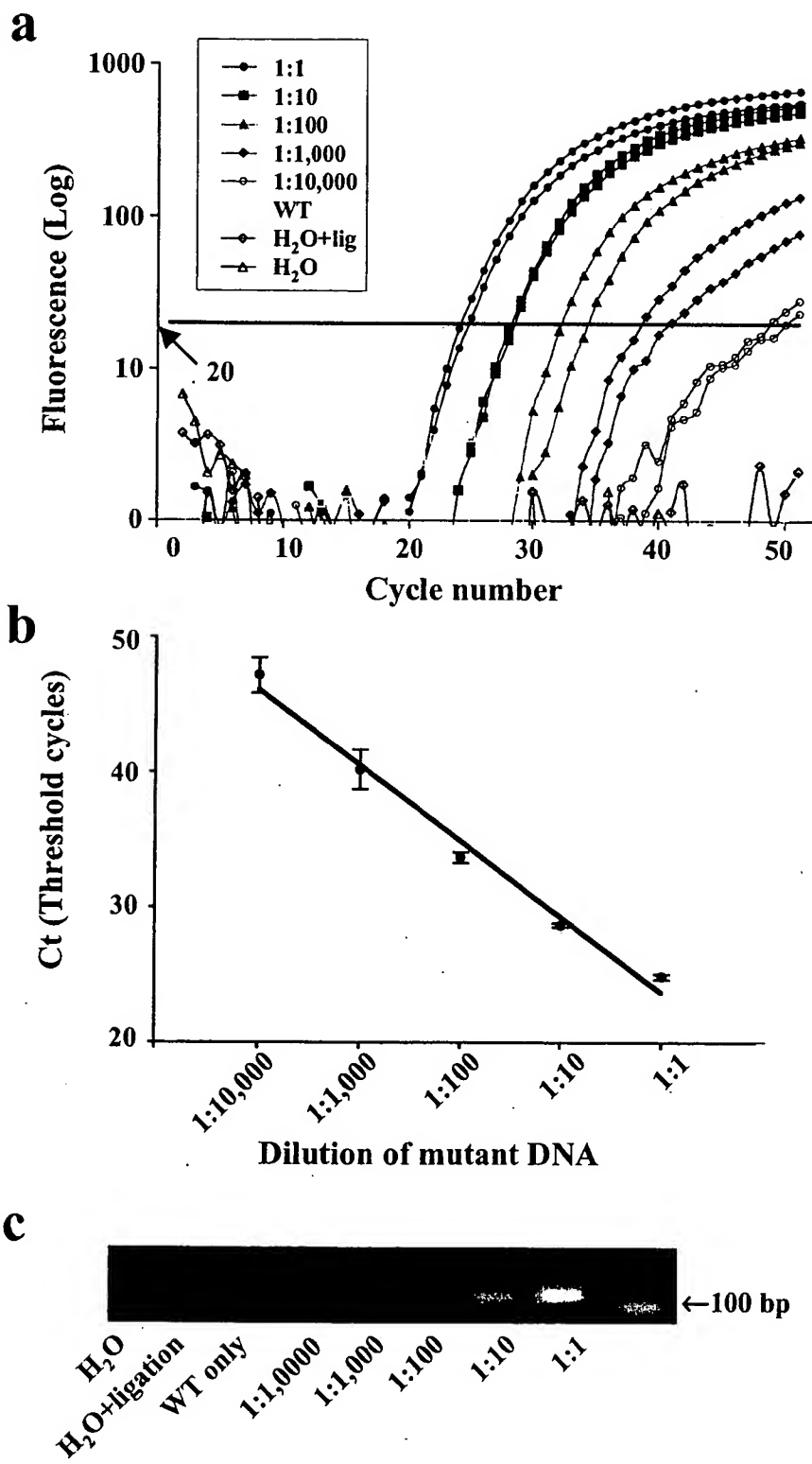


Fig 3. Shi et al.



## LigAmp: Sensitive Detection of Single Nucleotide Differences

Chanjuan Shi<sup>1</sup>, Susan H. Eshleman<sup>1</sup>, Dana Jones<sup>1</sup>, Noriyoshi Fukushima<sup>1</sup>, Li Hua<sup>1</sup>, Antony R. Parker<sup>1</sup>, Charles J. Yeo<sup>2</sup>, Ralph H. Hruban<sup>1,4</sup>, Michael G. Goggins<sup>1,2,3</sup>, and James R. Eshleman<sup>1,4,5</sup>

Departments of <sup>1</sup>Pathology, <sup>2</sup>Surgery, <sup>3</sup>Medicine and <sup>4</sup>Oncology  
Johns Hopkins University School of Medicine

**Keywords:** cancer, oligonucleotide ligation assay, RQ-PCR, Cepheid SmartCycler, real-time PCR, mutation, HIV-1, *KRAS2*, minority variant, multiplex.

**Abbreviations:** ARMS, Allele refractory mutation system; AS-PCR, allele-specific PCR; Ct, cycle threshold; OLA, Oligonucleotide ligation assay; PAGE, polyacrylamide gel electrophoresis; RFLP, restriction fragment length polymorphism; RQ-PCR, real-time quantitative PCR; SNP, single nucleotide polymorphism.

**Acknowledgements:** We acknowledge Drs. Bert Vogelstein, Ken Kinzler, Kathy Murphy, and Brian Wendelburg (Cepheid), in addition to Marsha Slater (Applied Biosystems), Kieran Brune, and Jeff Strain for helpful discussions. We thank Dr. John Mellors (Univ. of Pittsburgh) for providing plasmids containing HIV-1 molecular clones with and without the K103N mutation. This work was supported by R01 CA81439 from the National Cancer Institute, and the Maryland Cigarette Restitution Fund (to J.R.E.), by the HIV Prevention Trials Network (HPTN) sponsored by the NIAID, National Institutes of Child Health and Human Development (NICHD), National Institute on Drug Abuse, National Institute of Mental Health, and Office of AIDS Research, of the NIH, DHHS (U01-AI-46745 and U01-AI-48054), the Adult AIDS Clinical Trials Groups (NIH, Division of AIDS, NIAID), and R01-HD042965-01 (to S.H.E.), and by a Fellowship from the Canadian Institute of Health Research (to C.S.).

**<sup>5</sup>. Correspondence:**

James R. Eshleman, M.D., Ph.D.  
Associate Professor  
Department of Pathology  
Ross 632  
720 Rutland Ave.  
Baltimore, MD 21205  
[jeshlema@jhmi.edu](mailto:jeshlema@jhmi.edu)  
phone: 410-955-3511  
fax: 410-614-0671



**ABSTRACT**

Sensitive detection and accurate quantification of small numbers of mutant viruses and cells is a challenge in many research and clinical applications, particularly when a mutant DNA sequence differs from the corresponding wild-type DNA sequence at a single base. A novel method (LigAmp) was developed to detect single base differences. In LigAmp, two oligonucleotides are adjacently hybridized to a DNA template. One of the oligonucleotides is designed to perfectly match the target (e.g. mutant) sequence and also contains a foreign probe sequence. The two oligonucleotides are then ligated if the target is present, thereby converting the difficult-to-detect single base mutation in the target into a molecule with a completely unique probe site. Ligated products are detected using real-time quantitative PCR (RQ-PCR). In a model for sensitive detection of small numbers of cancer cells, LigAmp can detect *KRAS2* mutant DNA in wild-type DNA at a level of 0.01%-0.001%. As a model of early detection of cancer, *KRAS2* mutations were detected in pancreatic duct juice from patients with pancreatic cancer. LigAmp can also detect the K103N HIV-1 drug resistance mutation at a level of 0.01% in plasmid mixtures, and at a level of 0.1% in DNA amplified from HIV-1 in plasma samples. Detection in both systems is linear over a wide dynamic range (4-5 orders of magnitude). Moreover, reactions can be multiplexed, detecting different mutations, or both mutant and wild-type DNA, simultaneously. The LigAmp strategy provides sensitive and linear detection of single base mutations in genomic DNA and cDNA. This assay may find applications in the management of cancer and infectious diseases.

## INTRODUCTION

Single base mutations and polymorphisms play a role in the pathogenicity of many human diseases, including cancer. Viral drug resistance is also commonly associated with single base mutations in viral genomes. In addition to their role in pathogenicity, single base mutations can be effective markers for DNA-based diagnostic tests. Unfortunately, mutant cells and viruses are often present in clinical samples as minor populations, mixed with a vast excess of wild-type cells or viruses. Development of sensitive and accurate strategies to detect low levels of DNA or RNA with a specific mutation is an important challenge.

Several assays can detect single base differences in DNA or RNA, but their limit of detection is typically 0.1-1% (ratio of mutant/wild-type of 1/100-1/1000). Restriction length polymorphism (RFLP)/Southern blot assays typically have a limit of detection of 0.5-5% (1, 2). The oligonucleotide ligation assay (OLA), even with sensitive capillary electrophoresis detection, can only detect minor species at about 0.1-1% (3, 4). With allele-specific PCR (AS-PCR) or the amplification refractory mutation system (ARMS), two separate PCR reactions are used, each with a forward PCR primer that is perfectly matched to either the wild-type or mutant allele at its 3' end (5, 6). However, Taq polymerase, which is often used in amplification reactions, can still extend DNA from a mispaired primer. This limits the sensitivity of detection of AS-PCR and ARMS assays to approximately 1% (7, 8). Ligation chain reaction uses four oligonucleotides and ligase as an alternative to polymerase, but its limit of detection is approximately 0.1 to 1% (9). We recently attempted to use RQ-PCR and single nucleotide polymorphisms (SNPs) to monitor the level of bone marrow transplant engraftment in mixed chimeric DNA samples, but were only able to attain a limit of detection of 5-10% due to cross-hybridization of the probes (10). Other assays can detect single base mutations at lower levels (11-14). However, these methods often rely on the presence of unique DNA sequences adjacent to the target

mutations, and therefore cannot be generally applied. The present study was designed to develop a universal strategy for sensitive detection and accurate quantification of single base differences.

## MATERIALS AND METHODS

### DNA Isolation and Cell line Mixtures

Wild-type (HeLa cells) and *KRAS2* mutant (SW480 and LS513 cells) genomic DNA was extracted using the DNeasy Tissue Kit (Qiagen, Valencia, CA). Ten-fold serial dilutions of the mutant DNA (1.2 µg to 12 pg) were added to 1.2 µg wild-type DNA. Pancreas tumor frozen tissue sections and pancreatic duct juice were harvested at surgery and DNA was isolated as previously described ((15)) using the same DNeasy Tissue Kit. Approval from the Johns Hopkins Committee for Clinical Investigation was obtained for all experiments involving human subjects.

### *KRAS2* sequencing

Sequencing was performed on pancreatic cancer DNA or pancreatic duct juice DNA after PCR amplification of the *KRAS2* locus. The primers used for PCR amplification were 5'-GTAAAACGACGGCCAGG-GAGAGAGGCCTGCTGAAAA-3' and 5'-CAGGAAACAGCTATGACT-TGGATCATATTCGTCCACA-3', which contain 5' terminal M13 tails (underlined regions). Sequencing of the amplified DNA was performed using M13 forward or reverse primers, the BigDye Terminator 3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) and an ABI Prism 3100 Genetic Analyzer (Applied Biosystems).

### LigAmp oligonucleotides and probes

Oligonucleotides (gel-purified), and M13 forward and reverse primers (Table 1) were purchased from Invitrogen, Corp. (Carlsbad, CA). The *lacZ* and *16S rDNA* Taqman probes containing different

fluorophores and quenchers (Table 1) were purchased from Integrated DNA Technology (Coralville, IA).

#### *KRAS2* and *p53* oligonucleotide ligation

For *KRAS2* or *p53* analysis of cell line mixtures, ligation was performed directly on genomic DNA. For pancreatic duct juice samples, a region of *KRAS2* was first PCR amplified as described above. Sixty pg of this PCR product was used as a template for ligation. One pmol of the appropriate upstream mutant or wild-type oligonucleotide and 1 pmol of the common oligonucleotide were incubated with the DNA sample and 4 U *Pfu* DNA ligase in 1x *Pfu* Ligase Buffer (Stratagene, La Jolla, CA). The ligation conditions included denaturation at 95 °C for 3 min, followed by 90 cycles of 95 °C for 30 seconds and 65 °C for 4 min. Ligation reactions for wild-type and mutant alleles were initially performed in separate tubes and used directly for RQ-PCR without purification. Multiplex LigAmp was performed for mutant and wild-type *KRAS2* by including both upstream oligonucleotides in the reaction and reducing the concentration of the wild-type upstream oligonucleotide 100,000-fold to  $10^{-5}$  pmol. Multiplex LigAmp for the *KRAS2* and *p53* mutations was performed using the two mutant upstream and the two common downstream oligonucleotides, all at 1 pmol.

#### RQ-PCR

RQ-PCR amplification of ligated products was performed using a SmartCycler (Cepheid, Sunnyvale, CA), which was chosen for initial optimization since the module for each reaction can be independently controlled. Each reaction (25 µl) contained 5 pmol forward and 5 pmol reverse M13 primers, 6 µl of the unpurified ligation reaction, 12.5 µl platinum Quantitative PCR SuperMix-UDG (Invitrogen), and 2.5 pmol of the *lacZ* and/or *16S rDNA* probes. PCR reactions included pre-incubation at 50 °C for 2 min and 95 °C for 2 min, followed by 50 cycles of 95 °C for 10 seconds and 64 °C for

20 seconds. Following RQ-PCR analysis, PCR products were also analyzed by electrophoresis on 4-12% acrylamide gels (Novex® TBE gel, Invitrogen) and stained with ethidium bromide.

#### Population sequencing of plasma HIV-1

Plasma samples from three HIV-1 infected individuals were each diluted to a concentration of 100,000 copies/ml HIV-1 RNA using plasma from an individual without HIV-1 infection. Population sequencing (HIV-1 genotyping) was performed using the ViroSeq™ HIV-1 Genotyping System v2.0 (ViroSeq, Celera Diagnostics, Alameda, CA). In this system, RNA is extracted from HIV-1 and reverse transcribed with Moloney Murine Leukemia virus (MuLV) reverse transcriptase. A 40-cycle PCR using AmpliTaq Gold® DNA polymerase (Applied Biosystems) is performed, yielding a 1.8 kb PCR product. This PCR product includes regions that encode HIV-1 protease (amino acids 1-99) and HIV-1 reverse transcriptase (amino acids 1-324). PCR products are purified using spin columns and analyzed by agarose gel electrophoresis. DNA sequencing is performed using pre-mixed BigDye® terminator sequencing reagents (Applied Biosystems) with 7 different primers. Sequencing reactions are analyzed using an ABI PRISM 3100 Genetic Analyzer. Sequence data was analyzed using ABI Sequence Analysis v3.7 (Applied Biosystems) and the ViroSeq® HIV-1 Genotyping System software v 2.5. DNA sequence alignments were performed using MegAlign (DNASar, Madison, WI).

#### Oligonucleotide ligation for detection of the K103N mutation in HIV-1

For analysis in the LigAmp system, PCR products generated in the ViroSeq system (see above) were diluted in water to a concentration of 20 pg/μl. Ligation was performed using 100 pg of amplified DNA and the conditions listed above, except that 2 pmol of the upstream oligonucleotide (either wild-type or mutant). A plasma panel was also generated by mixing plasma from two of the individual described above (subjects 109 and 242, see Results). The ViroSeq system (see above) was used to

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extract and reverse transcribe HIV-1 RNA from the plasma mixtures, to amplify the resulting cDNA, and to purify the resulting PCR products. The PCR products were analyzed by agarose gel electrophoresis as described above, and then diluted in water and analyzed as described above.

#### HIV-1 oligonucleotide ligation using plasmid templates

Plasmids containing infectious molecular clones of HIV-1 with and without the K103N drug resistance mutation (mutant and wild-type) were generously provided by Dr. John Mellors (Univ. of Pittsburgh). A 1.8 kb PCR product that includes the region of HIV-1 reverse transcriptase that encodes codon 103 was amplified from the plasmids using the ViroSeq system (see above). The amplified DNA was cloned into the pCRII-TOPO® cloning vector using the TOPO TA Cloning® Kit, Version N (Invitrogen), and wild-type and mutant plasmids containing the 1.8 kb ViroSeq amplicon were isolated as previously described (16). Plasmids were sequenced using the ViroSeq system. Ten-fold serial dilutions of the mutant plasmid were prepared by mixing the mutant plasmid with the wild-type plasmid. Plasmid mixtures were prepared at a final concentration of 2 pg/μl. Ligation was performed using 10 pg of plasmid template as described above.

## RESULTS

### Overview of the LigAmp assay

RQ-PCR is an exciting, relatively new method in which PCR reactions are continuously monitored during amplification using fluorescent probes. It has a remarkably wide dynamic linear range, but has been difficult to apply to the detection of point mutations because probes that differ by a single base often cross-hybridize to the inappropriate wild-type template. Therefore, we sought a strategy that would convert single base differences into more distinctive molecules.

The first step of this strategy involves ligation of two oligonucleotides hybridized to a DNA template (Figure 1a). Each of the oligonucleotides contains a region specific to the target gene (green) and an M13 tail (blue). The M13 tails permit amplification of the ligated product with universal M13 primers in a subsequent universal RQ-PCR detection reaction. The upstream oligonucleotide also contains a region of unique foreign DNA (e.g. *lacZ* DNA, red) that serves as the binding region for a probe in the RQ-PCR reaction. The upstream oligonucleotide, which contains the probe-binding region, can be designed to hybridize to either the mutant or wild-type sequence. When an upstream mutant oligonucleotide is used (as shown in Figure 1a), the 3' end of the oligonucleotide is designed to perfectly match the corresponding mutant sequence in the target molecule. The same oligonucleotide should mis-pair at the 3' end when hybridized to a wild-type template, thereby preventing ligation (Figure 1b).

The second step of the strategy involves amplification and detection of the ligated DNA (Figure 1c). This step is independent of the specific gene or mutation targeted in the initial ligation step, and is universal in this sense. RQ-PCR is performed using M13 primers to amplify the ligated product. The resulting amplicons are then detected using a universal probe (e.g. *lacZ*). Note that both the M13 forward primer and the *lacZ* probe are designed to have the same polarity as the upstream ligation oligonucleotide. Therefore, the *lacZ* probe cannot bind to the ligation oligonucleotide. Binding of the

probe requires successful ligation of the two oligonucleotides (Figure 1a) followed by polymerization of the complementary (bottom) strand of DNA by Taq polymerase in the RQ-PCR step using the M13 reverse primer (Figure 1c). The RQ-PCR probe, which contains the *lacZ* sequence, a fluorophore (FL) and a quencher (Q), binds to the bottom strand of the amplified DNA. Subsequent extension of Taq polymerase from the M13 forward primer towards the *LacZ* probe allows the probe to be cleaved, and the fluorophore to be released and detected.

Since this strategy involves a ligation step followed by an amplification/detection step, we have designated it, "LigAmp". LigAmp can be performed using a mutant upstream oligonucleotide to detect and directly quantify only mutant DNA, as shown in Figure 1. Alternatively, a pair of mutant and wild-type upstream oligonucleotides can be used to separately or simultaneously detect and quantify mutant and wild-type DNA for relative quantification (not shown in Figure 1). In either case, the specificity of LigAmp relies on the differentiating power of a DNA ligase to ligate the upstream and downstream oligonucleotides only when they are both fully hybridized to the template DNA (i.e. no mismatch is present at either of the adjacent terminal nucleotides).

#### Detection of the *KRAS2* mutation in cell line DNA mixtures

To demonstrate initial proof-of-principal, we used a colon cancer cell line, SW480, which contains a *KRAS2* mutation (G35T, Gly12Val), and performed 10-fold serial dilutions of this mutant DNA into HeLa cell line DNA, which contains wild-type *KRAS2* alleles. After ligation of either a mutant or wild-type specific oligonucleotide to a common oligonucleotide, RQ-PCR was performed using the universal M13 primers and *lacZ* probe. In RQ-PCR, amplification occurs at different cycle numbers depending on the amount of template initially present. To quantify this, a fluorescence threshold is defined and the cycle at which this threshold is crossed is determined for each sample (cycle threshold, Ct). The Ct value is inversely correlated with the amount of template initially



present. Figure 2a shows that the mutant *KRAS2* DNA can be detected as a signal distinct from pure wild-type DNA even when present at a dilution as low as 1:10,000 (4-5 cycles difference in Ct). At a dilution of 1:100,000, the signal was still distinct from that of the wild-type DNA alone ( $p < 0.01$ , paired student *t*-test), though the Ct difference was only 1-2 cycles. Detection of the *KRAS2* mutant DNA was linear over the full range of dilutions tested (Figure 2b,  $r^2 = 0.99$ , least squares analysis). When the same amplified products detected in RQ-PCR in Figure 2a were analyzed by polyacrylamide gel electrophoresis (PAGE, Figure 2c), the intensity of the ethidium bromide-stained PCR product (predicted size 106 bp) correlated well with the level of input mutant DNA, and correlated inversely with the RQ-PCR Ct, as expected.

#### The LigAmp reaction can be multiplexed

Mutant and wild type *KRAS2* were simultaneously detected in a multiplex reaction by including both mutant and wild-type upstream oligonucleotides in the ligation reaction, and both *lacZ* and *16S rDNA* probes in the RQ-PCR step. To determine the sensitivity of detection of the mutant DNA in a multiplex format, mutant SW480 DNA was serially diluted into the wild-type HeLa DNA as above. As shown in Figure 2d, the signal reflecting the mutant DNA varied with input DNA concentration (including the 1:10,000 dilution), whereas the signal from the wild-type DNA was relatively constant (Ct approximately 34, red amplification curves). To test if we could detect two different mutations simultaneously in a single LigAmp reaction, we included oligonucleotides to detect both the *KRAS2* (G35A, Gly12Asp) and *p53* (G818A, Arg273His) mutations in the same ligation reaction. Two different colored fluorophore probes were used in the RQ-PCR step. The multiplexed LigAmp reaction readily detected those mutations when challenged with DNA containing either the *p53* mutation alone (Figure 2e) or the *KRAS2* mutation alone (Figure 2f).

Detection of tumor-specific *KRAS2* mutations in pancreatic juice

To test the potential of applying LigAmp in a clinical setting, we chose the early detection of pancreatic cancer as a model system. Mutations of *KRAS2* are found in ~90% of pancreatic cancers but their use as a marker of pancreatic cancer is complicated by the frequent mutation of *KRAS2* in patients with chronic pancreatitis and with precancerous lesions of the pancreas known as pancreatic intraepithelial neoplasias (PanINs) (17). One potential diagnostic application of LigAmp is to accurately quantify mutant *KRAS2* levels in biological fluids such as pancreatic duct juice as a means of distinguishing patients with pancreatic cancer from those who carry mutant *KRAS2* in their pancreatic secretions for other reasons. Indeed, one previous study suggested that while the mere detection of mutant *KRAS2* in pancreatic juice is not specific for pancreatic cancer (18), quantification of mutant *KRAS2* levels might more accurately differentiate pancreatic cancer from other conditions (19).

We first PCR amplified and sequenced the hotspot region of the *KRAS2* gene from four pancreatic cancer (PC) tissue samples following microdissection. At codon 12, we identified two cancers with G35A (Gly12Asp) mutations (PC7 and PC70), one cancer with wild-type *KRAS2* alleles (PC66), and one cancer with the G35T (Gly12Val) mutation (PC89) (Figure 3A, top row, arrows). We then sequenced the corresponding pancreatic duct juice (PJ) samples collected from the same patients at the time of surgery (Figure 3A, bottom row). In two of these samples (PJ7 and PJ89), small peaks in the sequencing electropherograms were detected that corresponded to the same mutations detected by sequencing in the tumor samples from the same patients (arrows).

We then performed *KRAS2* LigAmp assays using *KRAS2* PCR amplified DNA from the pancreatic duct juice samples. First, we confirmed that the amount of wild-type DNA was roughly equivalent in the four samples using a wild-type LigAmp reaction (Figure 3b). We then tested whether we could detect mutant *KRAS2* DNA containing any of the pancreatic cancer-associated mutations in

the pancreatic duct juice samples. When we used oligonucleotides specific for the G35A (Asp) mutation (Figure 3c), the two tumors known to contain that mutation amplified first (PJ7, blue and PJ70, red). Note that the blue curve (PJ7) amplifies significantly before the red curve (PJ70), and this correlates with the ability to see the mutation by direct DNA sequencing of this pancreatic duct juice sample (Fig 3a, bottom).

The pancreatic duct juice sample from the patient whose tumor contained a predominance of the G35T (Val) mutation (PJ89, green) amplified between the G35A mutation-bearing cancers and the negative (HeLa) control DNA. Detection of the G35A mutation in this sample is presumably due to either intra-tumor clonal heterogeneity of *KRAS2* mutations (20) or because this patient's pancreas also contains high grade PanINs, with the G35A *KRAS2* mutation that contribute DNA to the pancreatic duct juice. The data from analysis of pancreatic duct juice from the patient whose tumor had wild-type alleles (PJ66, gold) overlapped with the HeLa negative control DNA. In the LigAmp reaction for the G35T (Val) mutation (Figure 3d), the pancreatic duct juice corresponding to the patient with the *KRAS2* G35T-bearing tumor (PJ89, green) amplified before the others. This correlates with the presence of a small peak in the direct DNA sequencing of this pancreatic duct juice sample (Fig 3a, bottom). The two tumors containing G35A mutations (PJ7 and PJ70) were intermediate in this assay, and the wild-type tumor (PJ66) again overlapped with the negative control.

#### Detection of the K103N mutation in HIV-1 plasmids

As a second model system, we tested whether the LigAmp assay could be used to detect minority variants with antiretroviral drug resistance mutations in a population of wild-type HIV-1 viruses. We prepared mixtures of plasmids with wild-type (K=AAA) or mutant (N=AAC) sequences at codon 103 in HIV-1 reverse transcriptase. The K103N mutation is associated with resistance to the non-nucleoside reverse transcriptase inhibitor (NNRTI) class of antiretroviral drugs used to treat HIV-

1 infection. We first prepared 10-fold serial dilutions of the mutant plasmid by mixing it with the wild-type plasmid, keeping the final concentration of DNA constant. We then performed the LigAmp ligation reaction using a mutant upstream and a common downstream HIV-1 oligonucleotide (Figure 4). RQ-PCR was performed using the same M13 primers and *lacZ* probe that were used to detect the *KRAS2* mutation (see above). Using this approach, we were able to detect the K103N mutation in the plasmid mixtures at a dilution of 1:10,000 (0.01%, Figure 4). The wild-type plasmid was not detected (Figure 4a). Detection of the K103N mutation was linear over the full range of dilutions tested ( $r^2=0.96$ , least squares analysis, Figure 4b).

Similar experiments were performed using an upstream oligonucleotide specific for the wild-type sequence, which further confirmed the specificity of the LigAmp assay (data not shown). Both the mutant and wild-type upstream oligonucleotides contained an additional base substitution (A→G) at the third base from the 3' terminus of the upstream oligonucleotide (Figure 5d, the first base of codon 103, table 1), to enhance specificity of the oligonucleotides for their respective templates.

#### Detection of the K103N mutation in plasma from HIV-1 infected individuals

We next tested whether the LigAmp assay could be used to detect the K103N mutation in plasma samples from HIV-1 infected individuals. Plasma samples were obtained from three HIV-1 infected individuals (subjects 242, 842, and 109). HIV-1 genotyping (population sequencing) was first performed using the FDA-cleared ViroSeq HIV-1 Genotyping System (see Methods, Figure 5). This system is designed to detect the major population of viruses in a plasma sample. Genotyping with the ViroSeq system revealed the following sequences at codon 103 in HIV-1 reverse transcriptase: Subject 242: N=AAC (mutant), Subject 842: K=AAA (wild-type), Subject 109: K=AAR=AAA/G (wild-type) (Figure 5a-c, respectively). Sequences obtained for each sample were aligned with the sequences of the HIV-1 upstream wild-type and HIV-1 downstream common LigAmp oligonucleotides (Figure 5d).

The HIV-1 sequences in each of the samples differed from the sequences of the LigAmp oligonucleotides at 3 or 4 positions. The HIV-1 sequence variation in these samples, including the presence of nucleotide mixtures at some positions, is consistent with the natural genetic diversity of HIV-1 in infected individuals. DNA amplified from each of the three samples was analyzed using the LigAmp assay with both mutant and wild-type upstream HIV-1 oligonucleotides.

In the sample from subject 242, the K103N mutation represented the major sequence detected by population sequencing (Figure 5a, left). Analysis of this sample with the LigAmp assay using the mutant and wild-type upstream oligonucleotides produced Ct's of 31.6 and 38.9, respectively (Figure 5a, right). The difference between the Ct values obtained for this sample using the two different upstream oligonucleotides was 7.3 cycles. This suggests that HIV-1 variants with the K103N mutation represent the major viral population, consistent with results from population sequencing. In this sample, wild-type HIV-1 sequences were also detected. In RQ-PCR, a 10-fold difference in template concentration typically results in a 3.3 cycle difference in Ct, providing that the reaction is 100% efficient (21). This is consistent with the Ct's observed using the HIV-1 plasmid mixtures (Figure 4). Therefore, the estimated prevalence of wild-type HIV-1 in this sample based on the Ct differences is between 1% and 0.1%. In the samples from subjects 842 and 109, the wild-type codon represented the major sequence detected by population sequencing (K=AAA in sample 842, and K=AAR=AAA/G in sample 109, Figure 5b and 5c, left). In both of these samples, the Ct obtained using the wild-type upstream oligonucleotide was higher than the Ct obtained using the mutant oligonucleotide, consistent with the genotyping results (Figure 5b and 5c, right). In both samples, RQ-PCR using the mutant upstream oligonucleotide did achieve threshold; this is in contrast to results obtained using a pure wild-type plasmid (Figure 4), and suggests that HIV-1 containing the K103N mutation was present as a minority variant in both samples. The difference in the Ct's obtained using the mutant and wild-type

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upstream ligation oligonucleotides was 8.3 for subject 842 and 10.3 for subject 109. The estimated prevalence of the mutant virus was between 1% and 0.1% for both samples.

#### Detection of the K103N mutation in a plasma dilution panel

Additional experiments were performed using a panel of plasma samples prepared by serially diluting plasma from subject 242 (mostly mutant) with plasma from subject 109 (mostly wild-type). The percentage of plasma from subject 242 in the panel samples was 100%, 10%, 1%, 0.1%, 0.01% and 0%. The HIV-1 RNA copy number of each sample in the plasma panel was the same (50,000 copies/ml HIV-1 RNA). Each of the individual plasma panel samples was independently subjected to HIV-1 RNA extraction, reverse transcription, and PCR amplification using the ViroSeq system. The resulting PCR products were adjusted to a final concentration of 20 pg/ $\mu$ l for analysis in the LigAmp assay. Using these plasma mixtures, the LigAmp assay detected the mutant DNA in the mixtures in a linear fashion, down to a dilution of 1:1,000 (0.1%,  $r^2=0.998$ , least squares analysis, Figure 6). Similar Ct's were obtained using the 1:1,000 dilution (Figure 6), further 10-fold dilutions (data not shown), and plasma from subject 109 alone (Figure 6), suggesting that plasma from subject 109 contained a minority population of viruses with the K103N mutation. Those results suggest that HIV-1 with the K103N mutation is present in the sample from subject 109 at a level of approximately 0.1%, consistent with results described above (Figure 5c).

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## DISCUSSION

In this report, we demonstrate that the LigAmp strategy is highly sensitive and specific, and can quantitatively detect low levels of DNA containing a single base mutation in the presence of a vast excess of wild-type DNA. A sensitivity of  $10^{-4}$  to  $10^{-5}$  was easily achieved for detection of a point mutation in genomic DNA and HIV cDNA. For both of these target molecules, LigAmp was highly reproducible, both within and between runs. Based on previous estimates that each human cell contains approximately 6 pg genomic DNA (12), the LigAmp assay should detect as few as 2-20 molecules of mutant *KRAS2* in the presence of vast excess of normal genomic DNA. A low-level, non-specific signal was observed using pure wild-type genomic DNA as template, but the Ct for that sample was clearly distinct from the signal obtained using a  $10^{-4}$  dilution of the mutant DNA. This non-specific signal is most likely generated during the ligation step, since it is generally not seen without the addition of ligated products to the RQ-PCR reaction. Experiments are in progress to determine whether this low-level activity is arising from non-specific ligation occurring despite the mispair or from template-independent ligation. The RQ-PCR detection step of the LigAmp assay employs a probe that hybridizes to a foreign DNA sequence (e.g. *lacZ*) introduced into the ligated product. This minimizes the potential for direct detection of the wild-type sequence, making this method more specific than traditional RQ-PCR based assays (10).

Previously reported strategies that are highly sensitive for detection of point mutations (see Introduction) are generally more complex than the LigAmp assay and less quantitative. Also, since no specific sequence context is required for the ligation step, and since the M13 primers and detection probes are universal in the RQ-PCR step, this method should be easily adapted for detection of any point mutation or single base difference. We also demonstrate that one can also perform the ligation step of LigAmp as a multiplex reaction for simultaneous detection of either mutant and wild-type or

multiple different target mutations. In this regard, other investigators have demonstrated the ability to simultaneously ligate up to forty oligonucleotide pairs (22).

For many cancer diagnostic applications, one would like to detect specific mutations that are common to many cancer types (e.g. *KRAS2*, *p53*, *BRAF* mutations). Several exciting new initiatives with very large potential clinical impact in cancer research are underway. For example, Dr. Sudhir Srivastava and the National Cancer Institute initiated an Early Detection Research Network (23) to develop early detection for common cancers, both for families at increased risk and for the general population (24, 25). Another exciting area of research is the new field of "molecular relapse" monitoring. In this setting, a cancer patient in remission is monitored for recurrence of cancer and re-treated when small numbers of cancer cells are detected using sensitive molecular strategies (26). Finally, a sensitive point mutation detection strategy might permit the use of SNPs to monitor bone marrow transplant engraftment (10).

Pancreatic cancer is an especially lethal disease, due in large part to the advanced stage at presentation. Therefore, the early detection of pancreatic cancer is critical for improving survival rates. Since approximately 90% of pancreatic cancers carry *KRAS2* mutations, most of which are located at codon 12, *KRAS2* has been extensively investigated as a marker of pancreatic cancer (27). Indeed, *KRAS2* mutations can be detected in endoscopically collected pancreatic duct juice, in plasma and in stool, could in theory be a potential diagnostic tool for pancreatic cancer (28). However, several studies have reported that *KRAS2* mutations are often detected in pancreatic duct juice and stool from patients without cancer, such as those with chronic pancreatitis and those with PanINs (29). For this reason, qualitative detection of mutant *KRAS2* alone is not an accurate predictor of pancreatic cancer. We hypothesize that pancreatic cancers will shed more mutant DNA than patients with chronic pancreatitis or those with PanINs alone. Indeed, in one study, quantification of mutant *KRAS2* levels in pancreatic duct juice was a more accurate predictor of pancreatic cancer than the mere detection of mutant *KRAS2*



(19). The need to be able to accurately differentiate pancreatic cancer from chronic pancreatitis is important because the two diseases can co-exist, or may have similar symptoms and current tests are not sufficiently able to distinguish these two diseases in clinical practice. Our results show that LigAmp can accurately quantify single base mutations, either relatively or absolutely, with a large linear range. The accurate quantification of mutant DNA by LigAmp may prove useful for the early detection of pancreatic cancer, because of quantitative differences in the levels of *KRAS2* mutant DNA shed into pancreatic duct juice by benign and malignant lesions. Moreover, detection of *KRAS2* in combination with other mutations (e.g. in *p53*) may increase both the sensitivity and specificity of LigAmp for detection of pancreatic cancer and its precursor lesions.

Technologies for sensitive detection of point mutations are also potentially important for management of patients with HIV-1 infection. HIV-1 infected patients typically harbor a swarm of genetically-related viral variants. Use of antiretroviral drugs to prevent and treat HIV-1 infection can select for drug-resistant variants that have point mutations in the HIV-1 protease and reverse transcriptase coding regions. Most HIV-1 genotyping assays used to identify drug resistance mutations are designed to detect the major viral population, and have relatively low sensitivity for minority variants. A recent blinded, multi-center study compared the sensitivity of different methods to detect minority variants of HIV-1 with the K103N drug resistance mutation that were present at different levels in laboratory-prepared viral stocks (30). Two allele-specific PCR (AS-PCR) assays and a yeast hybrid assay specific for HIV-1 reverse transcriptase could detect the mutation at <1%. One AS-PCR assay detected the mutation as low as 0.1%. Other assays that were less sensitive included an RT-PCR assay with allele-specific hybridization, two oligonucleotide ligation assays, single genome RT/PCR and sequencing, and RT/PCR with pyrosequencing, and RT/PCR with direct sequencing of the bulk population using two FDA-cleared HIV-1 genotyping assays. The interest in developing sensitive methods for detection of HIV-1 minority variants is growing, following the report of a study that

suggests that the presence of such variants can influence treatment response (31). Using plasmid mixtures, LigAmp was able to detect the K103N mutation in dilutions as low as  $10^{-4}$  (0.01%). The LigAmp assay detected the K103N mutation at a level of approximately 0.1% in two patients with HIV-1 infection who had only the wild-type sequence detected with an FDA-cleared genotyping assay. It is difficult to define the limit of sensitivity of the LigAmp assay using plasma samples obtained directly from HIV-1 infected individuals, since all such individuals may harbor minority variants with antiretroviral drug resistance mutations, even prior to drug exposure (32, 33). In one study, the calculated prevalence of the Y181C mutation was 7 and 133 per 10,000 copies of HIV-1 RNA in two untreated patients (32). We are currently optimizing LigAmp for detection and quantification of minority drug resistance variants of HIV-1 in plasma samples.

LigAmp may also have applications in genetics. One could envision multiplex assays for panels of mutations (e.g. the common *CFTR* mutations) for population screening where the goal would be to determine whether a particular individual is wild-type or not. Subsequent testing would be required to determine which specific mutation is present. Another application might be prenatal diagnosis, to determine whether a fetus carries a known paternal point mutation (34). Using sensitive assays such as LigAmp, it may be possible to detect such mutations in a small number of fetal cells in maternal peripheral blood, thereby avoiding the need for amniocentesis or chorionic villous sampling procedures that carry some (albeit low) risk of fetal loss (35, 36).

In summary, LigAmp is a universal point mutation detection strategy that is highly sensitive and quantitative and is therefore likely to find many potential cancer, infectious disease and genetics applications.

**FIGURE LEGENDS**

Figure 1: Overview of the LigAmp assay. The LigAmp assay includes two steps: template-dependent ligation of two oligonucleotides (Step 1) and detection and quantification with RQ-PCR (Step 2). Details of the LigAmp assay are described in the Results section.

Figure 2: Detection of genomic DNA containing the *KRAS2* mutation. **a.** Representative RQ-PCR amplification curves (in duplicate) of *KRAS2* mutant SW480 DNA serially diluted into wild-type *KRAS2* HeLa DNA. The “H<sub>2</sub>O + lig” sample is a water control that was subjected to both the ligation and RQ-PCR steps; the “H<sub>2</sub>O” sample was only subjected to the RQ-PCR step. WT=wild-type DNA only. **b.** The mean Ct values (four independent amplifications) were plotted against the dilution of mutant DNA. **c.** PAGE of the DNA products produced in RQ-PCR. A 100 bp DNA size marker is shown on the right. **d.** Multiplex detection of mutant and wild type *KRAS2* (in duplicate). *KRAS2* mutant SW480 DNA was 10-fold serially diluted into HeLa DNA, and the signals for mutant and wild-type *KRAS* were simultaneously detected in RQ-PCR using *lacZ* and *16S rDNA* probes, respectively. **e, f.** Multiplex LigAmp for *KRAS2* and *p53*. Detection of the *p53* G818A mutation in SW480 DNA (**e**), and the G35A *KRAS2* mutation with serially diluted LS513 DNA (**f**) using multiplexed ligation oligonucleotides.

Figure 3: Detection of mutant *KRAS2* sequences in pancreatic duct juice from pancreatic cancer patients.

**a.** *KRAS2* DNA sequencing results from pancreatic cancer tissue samples (PC, top row) and the corresponding pancreatic duct juice samples obtained from the same patients at the time of surgery (PJ, bottom row). The electropherogram displays the antisense sequence. Antisense sequence text (bottom strand, left arrowheads) is in a 5' to 3' orientation and the corresponding sense sequences (top strand,

right arrowheads) are in a 3' to 5' orientation. The three bases for codon 12 are boxed. Peaks representing nucleotide mixtures at codon 12 are indicated below each electropherogram (arrows), and the mutant bases are underlined in the sense sequence. **b-d.** LigAmp detection of wild-type (WT) sequences and of the G35A (Asp) and G35T (Val) mutations, respectively, in four pancreatic duct juice samples. HeLa DNA (HeLa) was included as a control in Figures 3c and 3d, but was not included in the experiment shown in Figure 3b.

Figure 4: Detection of the HIV-1 K103N mutation in plasmid mixtures. **a.** Representative RQ-PCR amplification curves (in duplicate) of K103N mutant plasmid serially diluted with wild-type HIV-1 plasmid. WT=wild-type plasmid alone. Controls (H<sub>2</sub>O+lig and H<sub>2</sub>O) are described in the legend for Figure 2. Note that 1:10,000 dilutions of the mutant plasmid are clearly distinct from the pure wild-type plasmid, which doesn't achieve threshold. **b.** The mean Ct values (six independent amplifications) were plotted against the relative concentration of mutant plasmid. **c.** PAGE of the DNA products produced in RQ-PCR. A DNA size marker is shown on the right.

Figure 5. Population sequencing and LigAmp analysis of HIV-1 in plasma samples. **a-c, left:** Plasma HIV-1 from three individuals was analyzed using the ViroSeq HIV-1 Genotyping System, which is designed for population (bulk) sequencing of PCR-derived HIV-1 amplicons (see Methods). Electropherograms show sequences near codon 103 in HIV-1 reverse transcriptase. Arrowheads indicate the orientation of sense and anti-sense sequencing primers used to produce each electropherogram. The nucleotide sequence from an HIV-1 reference strain (HXB2, "Ref") is shown above the corresponding nucleotide sequence of each sample. The amino acids encoded by the reference sequence (above) and the sample sequence (below) are shown at the top of each panel using the single letter code. The nucleotide at the third position of codon 103 is boxed in the nucleotide

sequence from each sample. A mixture of nucleotides (A and G = R) is present at this position in the sequence from Subject 109 (c); the lower case designation (r) indicates that the nucleotide sequence was modified during review (editing). **a-c** (right): Representative RQ-PCR amplification curves from each of the three plasma samples are shown (subjects 242, 282, and 109, respectively). Curves were generated using either the HIV-1 upstream mutant oligonucleotide (Mut, red) or the HIV-1 upstream wild-type oligonucleotide (WT, blue). **d**: Nucleotide sequences obtained for each of the three plasma samples using the ViroSeq system are aligned with the sequences of the HIV-1 upstream WT and HIV-1 downstream common oligonucleotides. The region of sequence corresponding to each of the oligonucleotides is indicated at the bottom (arrows). A consensus sequence is shown at the top. The position of codon 103 is indicated (bracket). Nucleotides at the third position of codon 103 are boxed. An A → G nucleotide substitution was introduced into the upstream wild-type and mutant oligonucleotides to enhance specificity of the ligation reaction (underlined). Dots indicate nucleotides that match those in the consensus sequence. Nucleotides that differ from the consensus sequence are shown. IUB codes are used to indicate the detection of nucleotide mixtures at a given position. Nucleotides detected at the mixture positions are as follows: The major peak at all but one of the positions with A+G mixtures (R) was A. At position 37 in the primer alignment sequence from subject 842, the major peak was G. The major peaks at the position with A+C (M) and A+T (W) mixtures were A and T, respectively. The peaks at the positions with Y (T+C) were approximately equal.

Figure 6. LigAmp analysis of mixtures of plasma samples from two HIV-1 infected individuals. **a**: Plasma from subject 242 (mostly HIV-1 with the K103N mutation) was serially diluted with plasma from subject 109 (mostly wild-type HIV-1, see text). DNA was amplified from the plasma mixtures using the ViroSeq system (see Methods and Results). The LigAmp ligation reaction was performed using the HIV-1 upstream mutant oligonucleotide for detection of the K103N mutation. Representative

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RQ-PCR amplification curves from each of the samples in the plasma mixture panel are shown (left). The PCR negative sample (PCR neg) represents analysis of a sample of RNA diluent (ViroSeq control with no HIV-1 RNA) processed in parallel in the ViroSeq system. The H<sub>2</sub>O+lig control is described above. **b.** The Ct values were plotted against the dilution of sample 242 (see text). The Ct of the sample of plasma from subject 109 only (arrow) was similar to the Ct obtained with a 1:1,000 dilution of plasma from subject 242.

Table 1. Oligonucleotides and probes

<i>KRAS2</i> Ligation Oligos	<i>KRAS2</i> G35T mutant upstream	5'- <u>ACTGTAAAACGACGGCCAGTGT-TCCCCTCAA</u> ACTG GCAGATGCACG-C-TTGTGGTAGTTGGAGCTGT*-3'
	<i>KRAS2</i> G35A mutant upstream	5'- <u>ACTGTAAAACGACGGCCAGTGT-TCCCCTCAA</u> ACTG GCAGATGCACG-C-TTGTGGTAGTTGGAGCTGA*-3'
	<i>KRAS2</i> wild-type upstream	5'-A- <u>CTGTAAAACGACGGCCAGTGT-CGTATTACCGCG</u> GCTGCTGGCAC-C-TTGTGGTAGTTGGAGCTGG*-3'
	<i>KRAS2</i> common downstream	5'-PO <sup>4</sup> - <u>TGGCGTAGGCAAGAGTGCC-TGGTCATAGC</u> <u>TGTTTCCTGCA</u> -3'
<i>p53</i> Ligation Oligos	<i>p53</i> G818A mutant upstream	5'- <u>ACTGTAAAACGACGGCCAGTGT-</u> CGTATTACCGCGGCTGCTGGCAC- GAACAGCTTTGAGGT $\square$ CA*-3'
	<i>p53</i> common downstream	5'-PO <sup>4</sup> - <u>TGTTTGTGCCTGTCCTGGGAG-</u> <u>TGGTCATAGCTGTTTCCTGCA</u> -3'

HIV-1 Ligation Oligos	HIV-1 mutant upstream	5'- <u>ACTGTAAAACGACGGCCAGTGT-TCCCCTCAA</u> <b>ACTG</b> <i>GCAGATGCACG-CGCAGGGTTAAAAAAG</i> <b>G</b> <b>A</b> C*-3'
	HIV-1 wild- type upstream	5'- <u>ACTGTAAAACGACGGCCAGTGT-TCCCCTCAA</u> <b>ACTG</b> <i>GCAGATGCACG-CGCAGGGTTAAAAAAG</i> <b>G</b> <b>A</b> A*-3'
	HIV-1 common downstream	5'- PO <sup>4</sup> - <u>AAATCAGTAACAGTACTGGATGTGGGTG-</u> <u>TGGTCATAGCTGTTTCCTGCA</u> -3'
Amplification Primers	M13 forward	5'-CTGTAAAACGACGGCCAGTG-3'
	M13 reverse	5'-TGCAGGAAACAGCTATGACCA-3'
Probes	<i>lacZ</i> probe	FAM-5'-TCCCCTCAA <b>ACTGGCAGATGCACG</b> -3'-BHQ-1
	<i>16S rDNA</i>	ROX-5'-CGTATTACCGCGGCTGCTGGCAC-3'-BHQ-2

Footnote for Table 1: Underlined: M13 primer binding regions. Italics: probe binding regions (*lacZ* or *16S rDNA*). Bold: target-specific regions. Asterisk: terminal bases with perfect homology to either the wild-type or mutant sequences. FAM: 6-carboxyfluorescein. ROX: 6-carboxy-X-rhodamine. BHQ: black hole quencher. Boxed base: An additional mis-pair in the upstream HIV-1 mutant oligonucleotide was introduced at the third base from the 3' end to improve the specificity of the assay (the usual mutant 3' terminal sequence is GAAC).



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Figure 1. Shi et al.

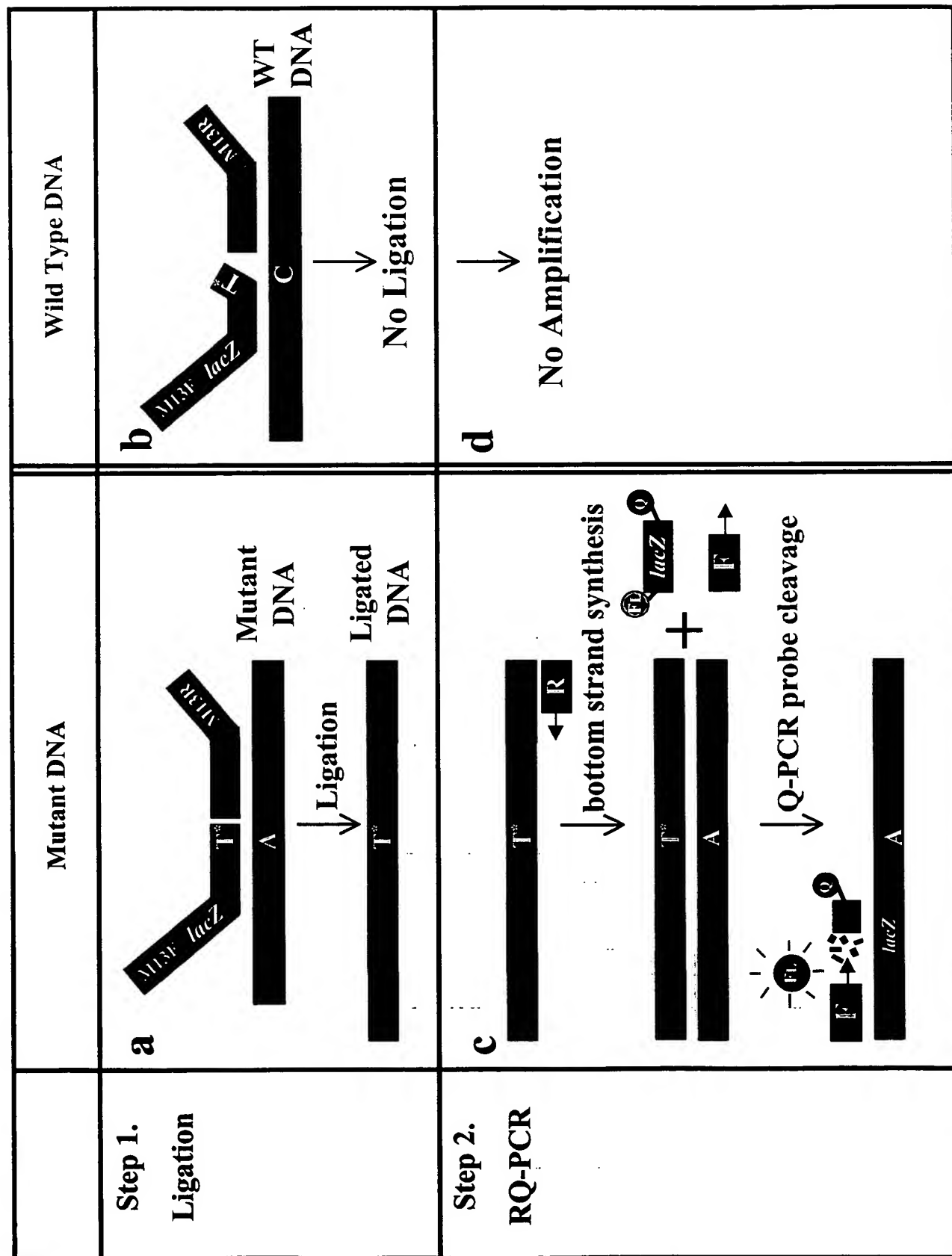


Figure 2, Shi et al.

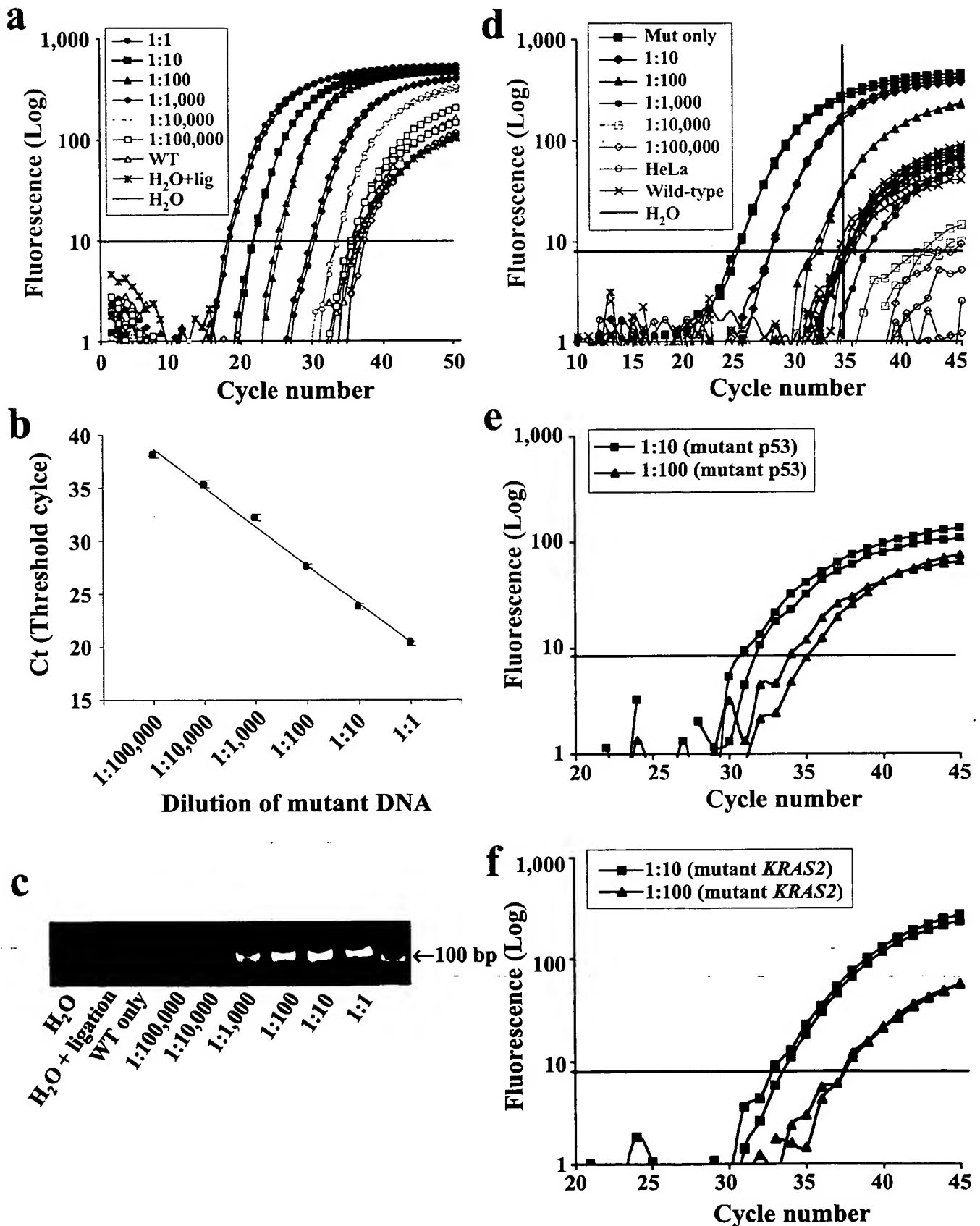


Figure 3, Shi et al

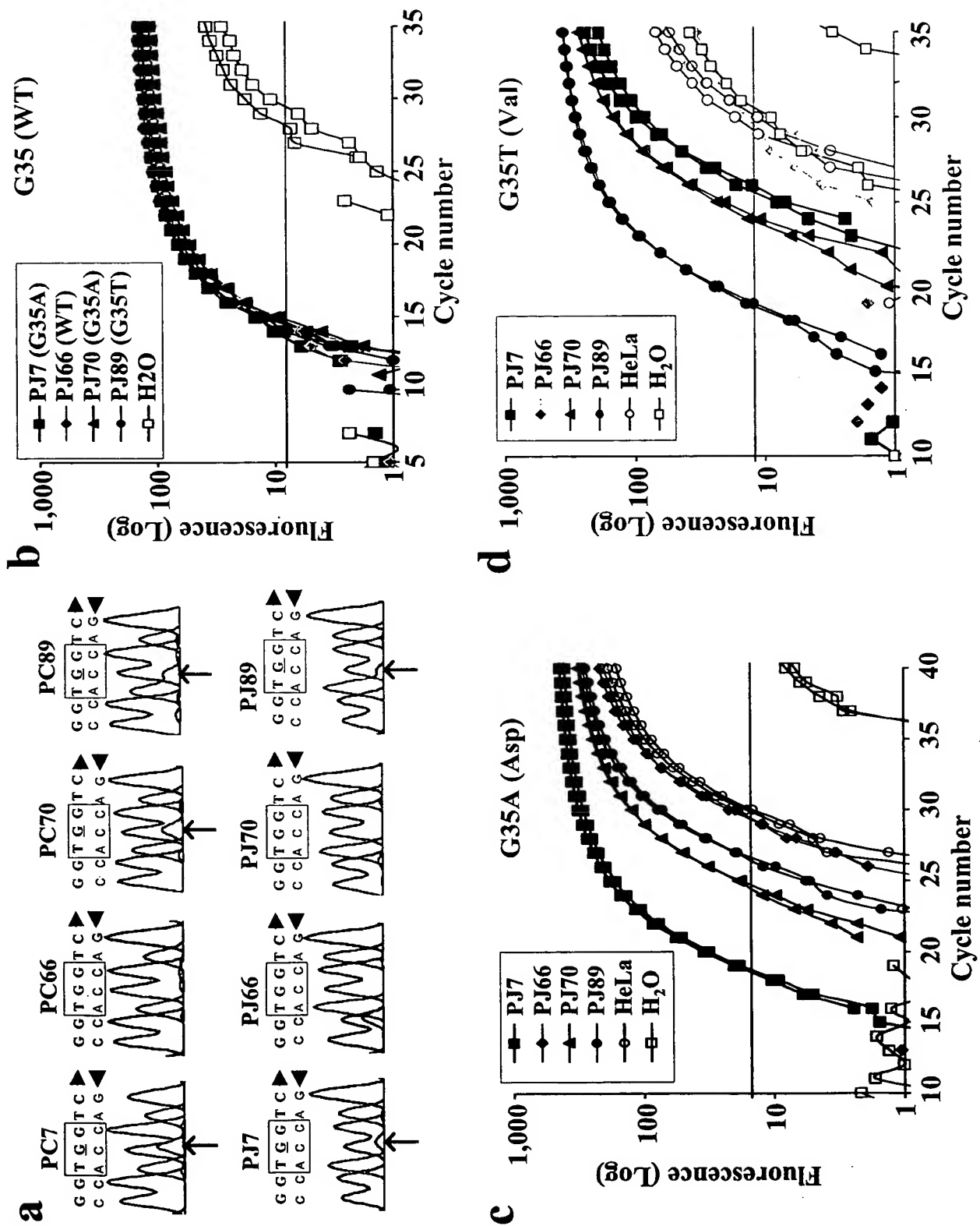


Figure 4, Shi et al.

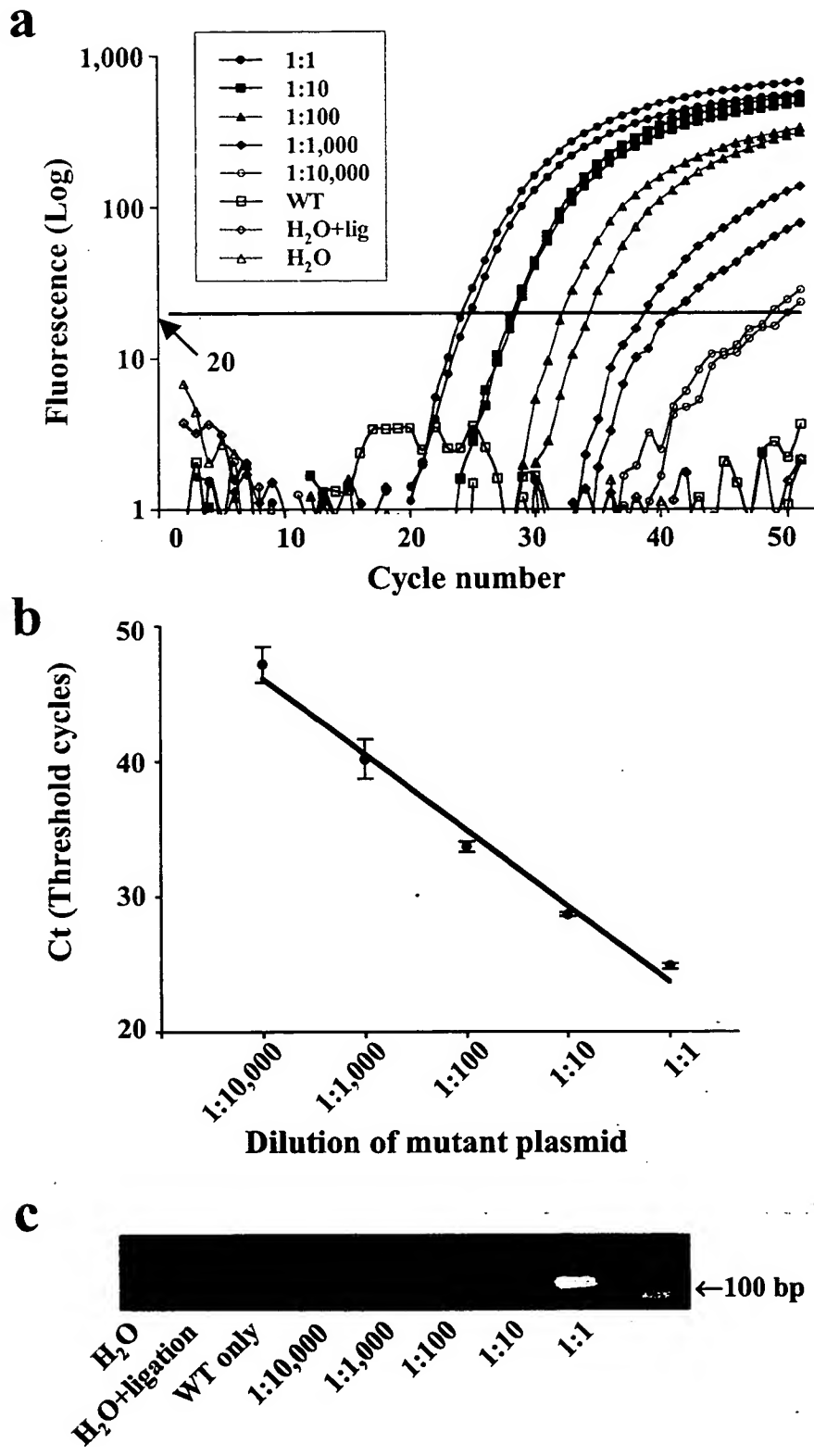


Figure 5, Shi et al.

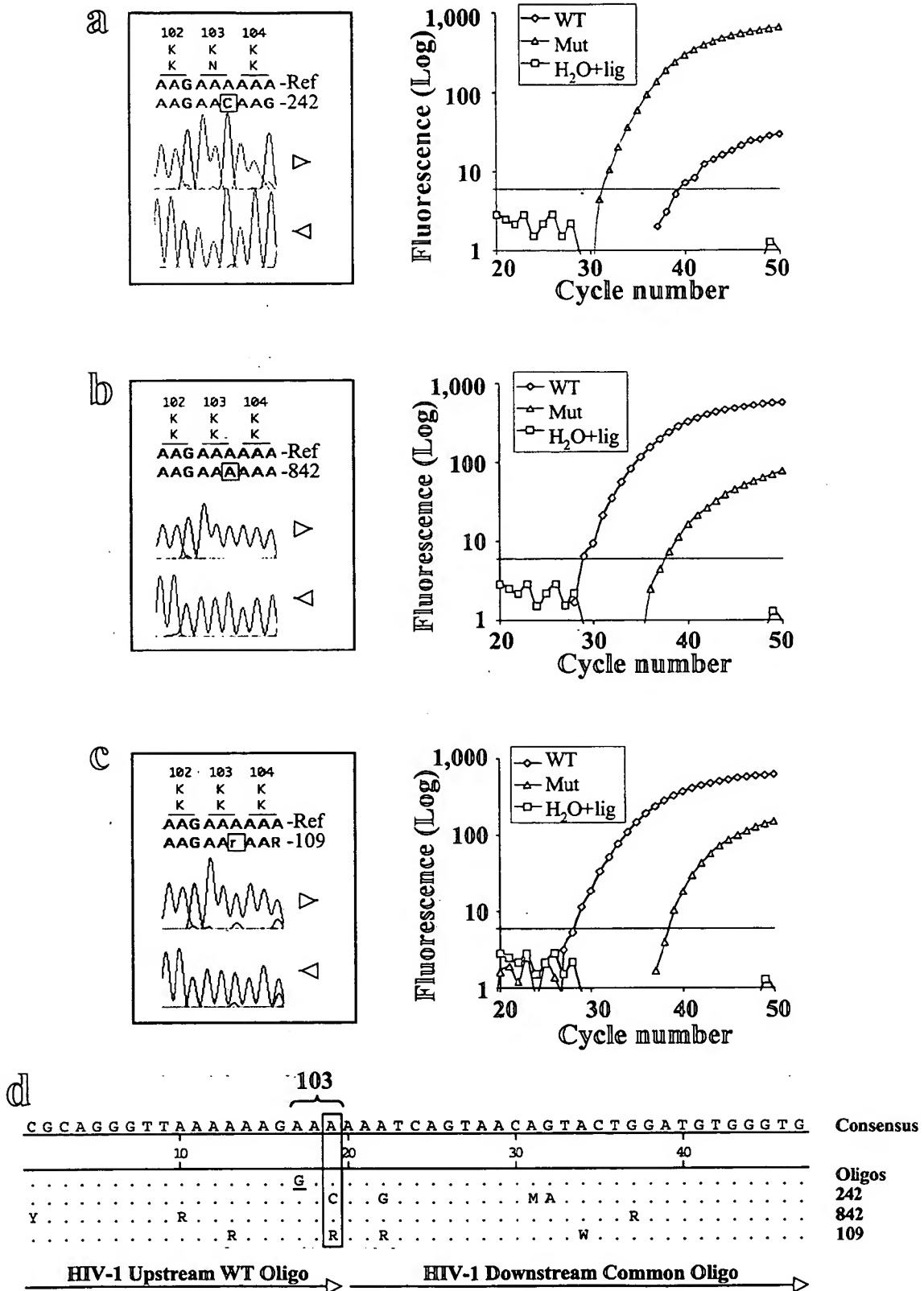


Figure 6, Shi et al.

